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A new angiotensin−converting enzyme inhibitor from *Peperomia pellucida* (L.) Kunth

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ABSTRACT

Objective: To isolate, identify, and evaluate a new angiotensin-converting enzyme inhibitor from *Peperomia pellucida* (L.) Kunth herbs.

Methods: A dried sample of *Peperomia pellucida* herb was successively macerated with *n*-hexane and ethyl acetate. The ethyl acetate extract solution was evaporated to obtain the crude extract. Vacuum liquid column chromatography and thin layer chromatography were performed to obtain two pure compounds. Then, both compounds were elucidated and identified using the spectroscopic method. Angiotensin-converting enzyme inhibitory activity studies of both compounds were determined using angiotensin-converting enzyme kit WST-1 with spectrophotometer microplate reader 96-well at 450 nm wavelength.

Results: Two bioactive compounds were successfully isolated from *Peperomia pellucida* herb, including a new compound of 2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene and pellucidin A. Both compounds demonstrated angiotensin-converting enzyme inhibitory activity, with IC_{50} values of 72 μM (27.95 μg/mL) and 11 μM (4.4 μg/mL), respectively.

Conclusions: In the present study, two active angiotensin-converting enzyme inhibitors were successfully isolated and purified from *Peperomia pellucida* which is used as an antihypertensive in traditional medicine, and support its use as an angiotensin-converting enzyme-inhibiting drug.

1. Introduction

Angiotensin-converting enzyme (ACE) is an essential enzyme that has a role in the regulation of blood pressure, as well as fluid and electrolyte balance in the human body, as it modulates the renin-angiotensin-aldosterone system[1,2]. ACE (a Zn^{2+}-

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binding metalloenzyme) increases the blood pressure when it is converted from angiotensin Ⅰ into the angiotensin Ⅱ, which acts a vasoconstrictor, thus contributing to hypertension[3]. Hypertension is a disease with reasonably high prevalence worldwide, causing blood pressure disorders and heart failure[4]. ACE is an ideal target for hypertension-controlling drugs[5,6], and several ACE inhibitors are widely available for the treatment of hypertension, including zofenopril, fosinopril, enalapril, ramipril, lisinopril, and captopril. However, all ACE inhibitors produce unpleasant side effects including fatigue, dizziness, and headaches[2].

Natural products have been the primary subjects of recent drug discovery. These studies have examined natural active compounds in an effort to discover new ACE inhibitors that are economical, safe to use, and of minimal side effects[5-7]. Since the development of an *in vitro* ACE inhibitory activity assay by Cushman and Cheung in 1971[2], drug discovery studies on ACE inhibitors from natural products have been more effective[8,9].

Peperomia pellucida (*P. pellucida*)(L.) Kunth herbs are one of the plant species that are traditionally used to lower blood pressure. *P. pellucida* herb extract has ACE inhibitor activity with an IC_{50} of 7.17 μg/mL[10] and the fraction and isolates (quercetin) have activity (IC_{50}) of 3.44 and 7.22 μg/mL[11], respectively. This herb contains secondary metabolites such as alkaloid, saponin, terpenoid, and polyphenol[12]. Several polyphenolic compounds have been successfully isolated including dillapiole[13], peperomins[14], pellucidin A[15], chromene[16], and quercetin[11]. However, until now, only quercetin has been successfully demonstrated to have ACE inhibitory activity[11]. *P. pellucida* herb has enormous potential as a herbal medicine, but so far it has not been commercially used as herbal medicine and is still considered as a weed mainly by farmers in oil palm plantations. Also, it has a poor yield value (mainly in the form of simplicial and extract). On the other hands, it still needs further scientific data to confirm its use as herbal medicine or traditional medicine.

The present study aimed to isolate and identify new bioactive compounds from *P. pellucida* as potential ACE inhibitors. We report the successful isolation of two compounds with ACE inhibitory activity: pellucidin A (which it was first identified from *P. pellucida* herb extract by Bayma and his colleague[15]) and a new compound of 2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene. To our knowledge, the *in vitro* ACE inhibition activity of both compounds has not previously reported.

2. Materials and methods

2.1. Reagents and apparatus

The reagents including *n*-hexane, ethyl acetate, chloroform, and methanol were purchased from PT. SmartLab Indonesia (West Java, Indonesia). Silica gel 60H (Merck), silica gel $GF_{254+366}$ (Merck), silica gel GF_{254} analytical (Merck) and preparative thin-layer chromatography (TLC) plates were purchased from Sigma-Aldrich (*via* PT. Elo Karsa, Indonesia). Captopril was obtained from Kimia Farma, Indonesia. An ACE Kit-WST1 was purchased from Doijindo Laboratories, Japan. The apparatus included 1-100 and 100-1000 μL micropipettes (Eppendorf, Germany), 96-well Microplate reader (VersaMax™ ELISA Microplate Reader, USA), Perkin-Elmer spectrum-100 FT-IR (Waltham, MA, USA), Shimadzu series 1800 spectrophotometer (Kyoto, Japan) UPLC-QToF-HR-MS XEV^{otm} mass spectrophotometer (Water, Milford, MA, USA), and an Agilent DD2 500 MHz NMR (^{1}H and ^{13}C; New Haven, USA).

2.2. Plants materials and sample preparation

P. pellucida herb materials were collected (March to May 2016) from the oil palm plantation at North Mamuju in West Sulawesi, Indonesia. The sample was identified at the Herbarium Bogoriense, Bogor, West Java, Indonesia. The voucher specimen was prepared as a dried powder sample and stored at a cool temperature (0-5 ℃) until use. The sample specimen was deposited at the Laboratory of Pharmacognosy–Phytochemistry, Faculty of Pharmacy, Universitas Indonesia, Depok, West Java, Indonesia.

2.3. Extraction, isolation and structure elucidation

A dried sample of *P. pellucida* herbs (3 kg) was successively macerated with *n*-hexane and ethyl acetate for 24 h. The ethyl acetate extract solution was evaporated using a rotary vacuum evaporator to obtain the crude extract. The ethyl acetate extract (8 g) was subjected to vacuum liquid column chromatography (170 mm×70 mm) using stationary phase of silica gel 60H (80 g) and 150 mL gradient elution of *n*-hexane: ethyl acetate (100:0, 80:20, 60:40, 40:60, 20:80, 0:100) and ethyl acetate: methanol (80:20, 60:40, 40:60, 20:80, 0:100), respectively to produce 11 fractions (A1-A11). The combined fraction of A2 and A3 (3.06 g) was subjected to vacuum liquid column chromatography using different gradient elution of *n*-hexane: ethyl acetate (80:20, 70:30, 60:40, 40:60, 0:100) and ethyl acetate: methanol (50:50), respectively to obtain 6 sub-fractions (B1-B6), and then each fraction was tested for ACE inhibitor activity. The most active sub-fraction of B1 (1.28 g) was recrystallized using 50% chloroform (in methanol) to obtain crystal powder compounds. Based on the TLC profile, the crystal powder compound contains two spots and then was separated using preparative TLC with eluent *n*-hexane/ethyl acetate (2:1) to obtain both pure compounds including compound 1 (11.33 mg) and compound 2 (6.24 mg), respectively. Structure elucidation was performed using spectroscopy method such as spectrophotometer UV-VIS, FT-IR, UPLC-QToF-MS/MS at Pusat Penelitian Kimia, Lembaga Ilmu Pengetahuan Indonesia Serpong, Tangerang, Banten, Indonesia, and NMR (^{1}H and ^{13}C) at Department of Chemistry, Faculty of Mathematics and Natural Sciences, Institut Teknologi Bandung, Bandung, West Java, Indonesia and Chemistry Division University of Malaya.

2.4. In vitro ACE inhibitory activity assay

The ACE inhibitory activity assay of 4,6,7-trimethoxy-1-(2,4,5-trimethoxybenzyl)-1H-indene (**1**) and Pellucidin A (**2**) was performed using an ACE Kit-WST1 (Doijindo Laboratories, Japan) according to the manufacturer's instructions and some literature[17-20]. This assay was conducted on both isolated compounds using captopril as a positive control (to compare the amount of 3HB formed by ACE activity) and a blank containing no ACE for method validation. The assay used 3-hydroxybutyryglycyl-glycyl-glycine as the substrate, and the absorbance was measured at 450 nm using a VersaMax™ ELISA Microplate Reader. Captopril was used as a positive control.

3. Results

3.1. Identification of 2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene

2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene: pale yellowish white amorphous powder, m.p. 153-155 ℃ (*n*-hexane/EtOAc); UV λ_{max} (logε) 203.0 (3.211), 230.0 (0.915), and 292.0 (0.484) nm; IR ν^{film} cm^{-1}: 2838, 2928, 2959, 2998, 1596, 1198, 1172, 1154, 1124, and 1106; ^{1}H-NMR and ^{13}C-NMR (Table 1); ESI-TOFMS [M + Na^{+}] *m/z* 409.162 (calc. $C_{22}H_{26}O_6$ [M^{+}] *m/z* 386.173). Based on the spectroscopic analyses, the structure of the compound isolated was determined as shown in Figure 1.

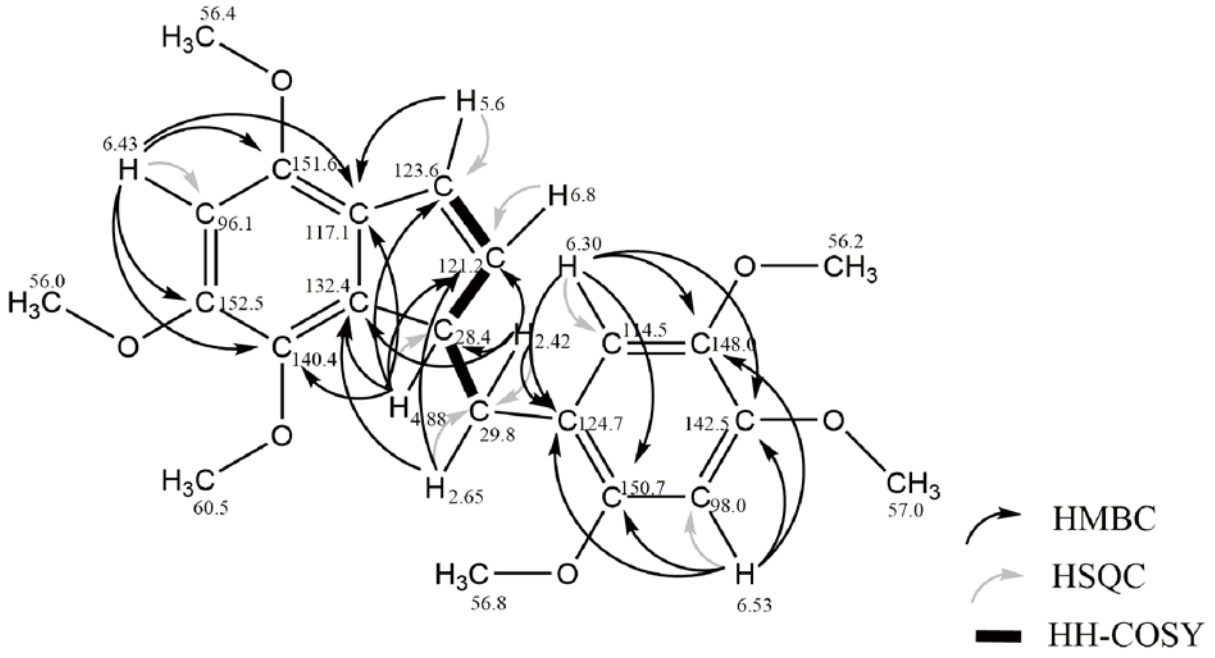

Figure 1. Two-dimensional NMR spectrum of new compound of 2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)- 1H-indene. HSQC: heteronuclear single quantum coherence; HMBC: heteronuclear multiple bond correlation; HH-COSY: homonuclear correlation spectroscopy.

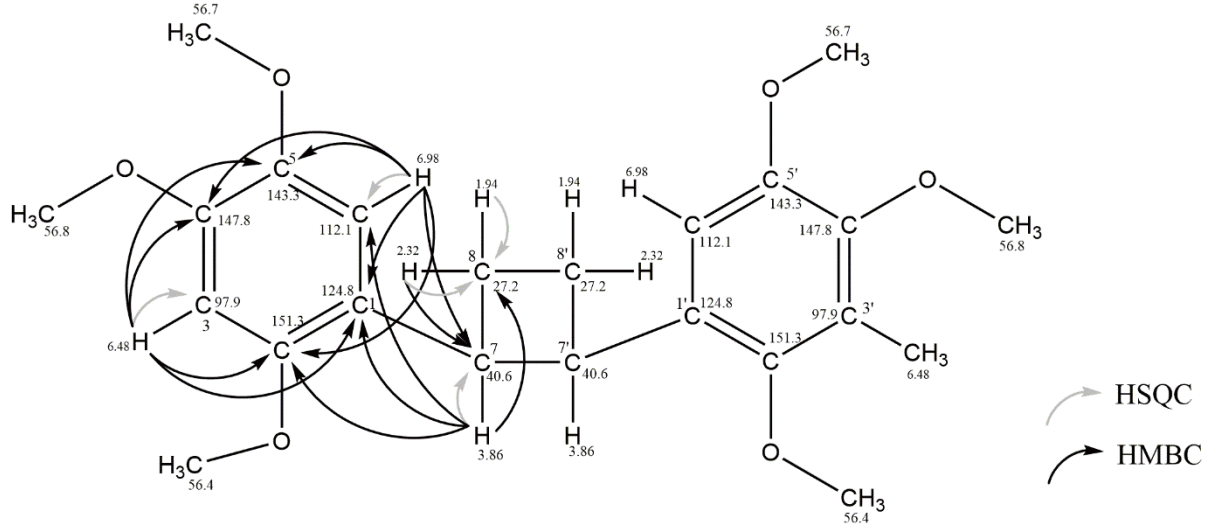

Figure 2. Two-dimensional NMR spectrum of pellucidin A. HSQC: heteronuclear single quantum coherence; HMBC: heteronuclear multiple bond correlation.

3.2. Identification of pellucidin A

Pellucidin A: $C_{22}H_{28}O_6$ with ESI-TOF-MS [M + Na] *m/z* 411.179 (calcd. $[M]^+$ *m/z* 388.189), showed FT-IR peaks at 1611, 1521, and 1489 cm^{-1}, revealing its aromatic ring; and at 2989 and 2936 cm^{-1}, showing the vibration of the C-H group. ^{1}H-NMR ($CDCl_3$, 500 MHz) and ^{13}C-NMR ($CDCl_3$, 500 MHz) (Table 2). Based on the spectroscopic analyses, the structure of pellucidin A was determined as shown in Figure 2 according to the literature[15].

3.3. ACE inhibitory activity

Table 3 shows that captopril had an IC_{50} value of 1.08×10^{-7} μM, equivalent to 2.35×10^{-11} μg/mL. Both isolated compounds (pellucidin A and the novel polyphenol) from *P. pellucida* had IC_{50} values of 72 μM (equivalent to 27.95 μg/mL) and 11 μM (equivalent to 4.4 μg/mL), respectively.

4. Discussion

This study successfully isolated, identified, and elucidated structurely two active compounds as an ACE inhibitor from *P. pellucida*. Both compounds were separated and purified using column and preparative TLC. Moreover, the determination of structure of the compounds were conducted using the spectroscopic method. ACE inhibitor activity was also analyzed using an ACE analysis Kit-WST1. Based on the best of our knowledge, one of them is a novel compound (compound **1**).

The molecular formula of novel compound (1): $C_{22}H_{26}O_6$ ($[M^+]$ *m/z* 386.173), showed FT-IR peaks at 2838, 2928, 2959, and 2998 cm^{-1}, revealing its C-H group (in the ranges of 2900–3100 cm^{-1}); peaks at 1198, 1172, 1154, 1124, and 1106 cm^{-1} showed the C-O group vibration (1085–1150 cm^{-1}); and a peak at 1596 cm^{-1} revealing its C=O aromatic group. ^{1}H-NMR spectra ($CDCl_3$, 500 MHz), showed three singlet protons (1H each, s) at δ_H 6.53, 6.44, and 6.30 ppm

Table 1. Chemical shift data of proton (500 MHz, $CDCl_3$), carbon (125 MHz, $CDCl_3$), and heteronuclear multiple bond correlation of 2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene.

Position	13C-NMR (δ_C, *J*)	1H-NMR (δ_H, *J*)	Heteronuclear multiple bond correlation
1	132.4	-	-
2	140.4	-	-
3	152.5	-	-
4	96.1	6.44 (1H,s)	C-2, C-3, C-5, C-6
5	151.6	-	-
6	117.1		
7	121.2	6.81 (1H, d, *J*=6.60 Hz)	-
8	123.6	5.67 (1H, m, *J*= 5.85 Hz)	C-6, C-7
9	28.4	2.65 (1H, dd, *J*= 8.28 Hz) & 2.42 (1H, m, *J*= 1.35 Hz)	C-1, C-2, C-6, C-7, C-8
10	29.8	4.88 (1H, d, *J*= 8.1 Hz)	C-1, C-8, C-9, C-11
11	124.7	-	-
12	150.7	-	-
13	98.0	6.53 (1H,s)	C-11, C-12, C-14, C-15
14	142.5	-	-
15	148.0	-	-
16	114.4	6.30 (1H,s)	C-11, C-12, C-14, C-15
(-OCH3)-2	60.5	3.39 (3H, s)	C-2
(-OCH3)-3	56.0	3.86 (3H, s)	C-3
(-OCH3)-5	56.4	3.86 (3H, s)	C-5
(-OCH3)-12	56.2	3.83 (3H, s)	C-12
(-OCH3)-14	56.9	3.57 (3H, s)	C-14
(-OCH3)-15	56.8	3.90 (3H, s)	C-15

Table 2. Chemical shift of proton (500 MHz, $CDCl_3$), carbon (125 MHz, $CDCl_3$), and heteronuclear multiple bond correlation of pellucidin A.

Position	13C-NMR (δ_C, *J*)	1H-NMR (δ_H, *J*)	Heteronuclear multiple bond correlation
1/1'	124.8	-	-
2/2'	147.8	-	-
3/3'	97.9	6.48 (1H, s)	C-1/1', C-2/2', C-4/4', C-5/5'
4/4'	151.3	-	-
5/5'	143.3	-	-
6/6'	112.1	6.98 (1H, s)	C-1/1', C-2/2', C-4/4', C-5/5', C-7/7'
7/7'	40.6	3.86 (1H, d, *J*= 4,15 Hz)	C-1/1', C-2/2', C-6/6', C-8/8'
8/8'	27.2	2.32 (1H, d, *J*=5.05 Hz) & 1.94 (1H, m, *J*=5.35 Hz)	C-7/7'
(-OCH3)-2/2'	56.8	3.75 (3H, s)	C-2/2'
(-OCH3)-5/5'	56.7	3.85 (3H, s)	C-5/5'
(-OCH3)-4/4'	56.4	3.85 (3H, s)	C-4/4'

Table 3. Results of angiotensin-converting enzyme inhibitor assay.

Samples	Concentration (μM)	Percentage inhibition (%)	Regression formulas (R^2)	IC_{50} (μM)
Captopril (positive control)	4.6×10^{-5}	88.73	Y=12.601X+183.94 (R^2 = 0.959)	1.08×10^{-7}
	2.3×10^{-5}	72.99		
	2.3×10^{-7}	52.50		
	4.6×10^{-8}	48.88		
	3.2×10^{-9}	27.78		
2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene	26.0	81.34	Y=81.233X–2.191 (R^2 = 0.972)	11
	21.0	68.87		
	16.0	64.07		
	10.3	41.14		
	5.2	24.79		
Pellucidin A	260	74.55	Y=46.824X–17.724 (R^2 = 0.995)	72
	130	62.08		
	64	49.81		
	32	34.23		
	16	17.99		

(aromatic proton) and five singlet protons (3H, s) at δ_H 3.90, 3.86, 3.86, 3.83, 3.57, and 3.39 ppm (methoxy proton). A doublet proton at δ_H 4.88 (1H, d, *J*=8.1 Hz), a doublet of doublet proton at 6.81 (1H, d, *J*=6.60 Hz), two multiplet protons at δ_H 5.67 (1H, m), 2.65 (1H, dd) and 2.42 (1H, m). ^{13}C-NMR spectra ($CDCl_3$, 125 MHz) showed 22 carbon signals at δ_C 28.4, 29.8, 56.0, 56.2, 56.4, 56.8, 56.9, 60.5, 96.1, 98.0, 114.4, 117.1, 121.2, 123.6, 124.7, 132.4, 140.4, 142.5, 148.0, 150.7, 151.6, and 152.5 ppm. The distortionless enhangcement by polarization transfer spectrum showed a methylene (δ_C 29.8) group, six methine (δ_C 28.4, 98.0, 96.1, 114.4, 121.2, and 123.6) groups, and six methoxyl (δ_C 56.0, 56.2, 56.4, 56.8, 56.9, and 60.5) groups. The homonuclear correlation spectroscopy spectrum showed correlations of the proton 4.88 (1H, d, *J*=8.1 Hz) with a methylene group (δ_C 29.8) and the proton 2.65 (1H, m) with C atom (δ_C 123.6) bond proton 5.67 (1H, m). The heteronuclear single quantum coherence spectrum showed correlations between proton and carbon at 6.44 (1H, s) with C (δ_C 96.1), 6.30 (1H, s) with C (δ_C 114.4), 6.53 (1H, s) with C (δ_C 98.0), 6.81 (1H, d, *J*=6.60 Hz) with C (δ_C 121.2), 5.67 (1H, m) with C (δ_C 123.6), 4.88 (1H, d, *J*=8.1 Hz) with C (δ_C 29.8), and 2.65 (1H, m) and 2.42 (1H, m) with C (δ_C 28.4). The heteronuclear multiple bond correlation spectrum revealed the correlation of signals in the aromatic carbon region at 6.53 (1H, s) and 6.30 (1H, s) with each other (δ_C 124.7, 142.5, 148.0, and 150.7), and 6.44 (1H, s) with each C atom (δ_C 117.1, 140.4, 151.6, and 152.5). Furthermore, the heteronuclear multiple bond correlation spectrum showed correlation of the signal proton at 5.67 (1H, m) with C atoms (δ_C 121.2 and 123.6), 4.88 (1H, d, *J*=8.1 Hz) with C atoms (δ_C 117.1, 121.2, 123.6, 132.4, and 140.4), and 2.65 (1H, dd, *J*=8.28 Hz) and 2.42 (1H, m, *J*=1.35 Hz) with C atoms (δ_C 28.4, 123.6, 124.7, and 132.4). Thus, the structure of 1 was elucidated as 2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene (a novel compound structure). Furthermore, compound 2 was also isolated and identified as pellucidin A by comparison of its spectroscopic data with this reported in the literature[15].

Since ACE was successfully isolated and identified from horse plasma (1954-1957), the discovery provides a significant impact in the development of the *in vitro* ACE inhibitor assay method[2], the research and discovery of new ACE inhibitor drugs have become more productive. Medicinal plant biodiversity is a valuable resource for drug discovery, and medicinal plant-based products may be explored to combat hypertension. In the present study, we successfully isolated two compounds from the ACE-inhibitory fraction of *P. pellucida* herb extract, namely pellucidin A and a new compound. Pellucidin A is a dimeric ArC_2 that was initially reported by Bayma and his colleague[15] and 2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene is a novel compound, the structure of which was established in the present study.

In the present study, the positive control (captopril) had an IC_{50} value of 1.08×10^{-7} μM, equivalent to 2.35×10^{-11} μg/mL. This result was similar to the results reported by Ibadallah and his colleagues[20] using the 3HB method (with an IC_{50} value of 1.0×10^{-11} μg/mL).

Both compounds show inhibitory activity against ACE, with IC_{50} values of 72 μM (equivalent to 27.95 μg/mL) and 11 μM (equivalent to 4.4 μg/mL), respectively. According to the inhibitory activity of these compounds (less than 50 μg/mL), both have the potential as pharmaceutical ACE inhibitors. However, two isolates show reduced activity compared to current ACE inhibitor drugs, although activity assays showed that 2,3,5-trimethoxy-9-(12,14,15-trimethoxybenzyl)-1H-indene had stronger ACE-inhibitory activity than quercetin compounds in the previous study[11]. Some studies have reported that other compounds have activities similar to those compounds in the present study, including compounds belonging to the phenolic group (gallic acid, vanillic acid, catechol pyrogallol), flavonoid group (quercetin, kaempferol, rutin, apigenin, epicatechin), and stilbene groups[21], and also flavonoid-rich extract from *Actinidia macrosperma*[22], and *Onopordon acanthium*[8].

Until present, three bioactive compounds as ACE inhibitors have been found from *P. pellucida* herbs, including quercetin[11], pellucidin A and a new compound of 2,3,5-trimethoxy-9- (12,14,15-trimethoxybenzyl)-1H-indene in this study. These compounds can be further developed into a pharmaceutical product as an antihypertensive herbal medicine with a green extraction approach

as reported in a previous study[23].

In conclusion, new ACE inhibitor compounds are successfully isolated and purified from *P. pellucida* in this study which is used as an antihypertensive in traditional medicine, and support its use as an angiotensin-converting enzyme-inhibiting drug. The compounds may be used as markers for the active ACE inhibitor extract/fraction of *P. pellucida*.

Conflict of interest statement

Authors declare that there are no competing interests.

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